*Oxis*Research™

A Division of OXIS Health Products, Inc.

BIOXYTECH® LPO-586™

Colorimetric Assay For Lipid Peroxidation
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21012

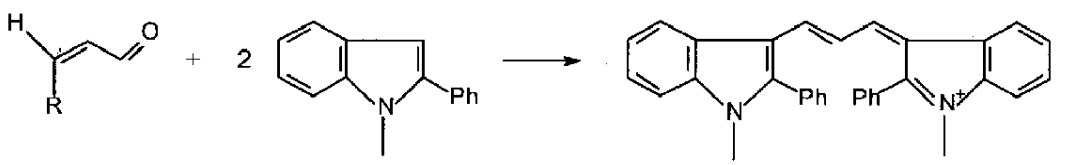
INTRODUCTION

The Analyte

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition. Measurement of malondialdehyde and 4hydroxyalkenals has been used as an indicator of lipid peroxidation (1). The LPO-586 method is designed to assay either MDA alone (in hydrochloric acid) or MDA in combination with 4-hydroxyalkenals (in methanesulfonic acid.)

Principles of the Procedure

The LPO-586™ assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm.



MDA : R = OH
4-hydroxyalkenal : R= hydroxyalkyl

Max = 586 nm

REAGENTS

Materials Provided (for 100 tests)

- Reagent R1 — N-methyl-2-phenylindole in acetonitrile, 3 X 18 mL
- Reagent R2 — Methanesulfonic acid, 1 X 16.5 mL
- 4-HNE Standard (S1) — 4-Hydroxynonenal, diethylacetal in acetonitrile, 1 X 1 mL
- MDA Standard (S2) — 1,1,3,3-Tetramethoxypropane in Tris-HCl, 1 X 1 mL

Materials Required But Not Provided

- Spectrophotometer for measuring absorbance at 586 nm from 0-2 absorbance units.
- Spectrophotometric cuvettes with a 1 cm optical path length.
- Water bath, set to control temperature at 45 ± 1°C.
- Disposable glass test tubes and stoppers compatible with acetonitrile, methanol, and acid.
- Methanol, 100% analytical or HPLC grade.
- HCl, 37%
- Butylated hydroxytoluene (BHT)
- Acetonitrile

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- Microcentrifuge
- Acid/acetonitrile resistant (e.g., polypropylene) microcentrifuge tubes.

Warnings and Precautions

- Do not smoke, eat or drink in areas where samples and reagents are handled.
- Wear disposable gloves when handling samples and reagents.
- Do not pipette reagents or samples by mouth.
- In case of accidental exposure of skin, mucous membranes or eyes to **R1** or **R2** reagents, thoroughly wash the exposed area with water.
- For *in vitro* use only. For research purposes only. Not for use in diagnostic procedures.

Reagent Storage and Handling

- Do not leave the reagent bottles open. Replace the caps as soon as the desired volume is removed.
- It is good practice to transfer the desired volume of reagents for an experiment to a clean glass test tube or other vessel and return the stock reagent bottles to 4°C.
- Do not allow the capped reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 4°C.
- New pipettes or pipette tips should be used when removing reagents from the stock bottle. Avoid contaminating the stock bottles as this could affect assay performance.
- If reagents are handled and stored properly as described above, they are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

Dilution of the R1 solution for use in the assay. Add one volume (6 mL) of 100% methanol to three volumes (18 mL) reagent R1. This solution (diluted R1) is stable for two days at 4°C. Do not leave the R1 reagent bottle uncapped (open to the atmosphere).

37% HCl Preparation

37% HCl is approximately 12 N acid reagent. This is the strength of concentrated HCl available from many chemical companies. **DO NOT DILUTE THE HCl PRIOR TO USE IN THE ASSAY.**

Sample Preparation

Note: Please read the appropriate **NOTES** sections before starting sample preparation procedure.

Preservative To Prevent Sample Oxidation

OXIS recommends that the researcher add BHT to a final concentration of 5 mM in the buffer prior to homogenization of tissue or cells. BHT can be made as a 100X stock 0.5 M solution in acetonitrile. If no antioxidant is added, new lipid peroxidation can occur during homogenization and biased values will result (2).

Concentration Of Tissue Homogenate/Sensitivity

Sample homogenates should be made as concentrated as possible. The concentration of protein in the homogenate should be determined. It is recommended that 0.2 mL of a homogenate containing 15-60 mg/mL protein be assayed for initial studies in a previously untested biological sample. For tissue culture cells, it is recommended that a sample derived from 10^7 cells be added to the LPO-586 assay (i.e., 0.2 mL of 1 X 10^7 per mL).

Sample Stability

Unless assayed immediately, samples should be frozen at -70°C to prevent loss of MDA and

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4-hydroxyalkenals (HAE) (3,4) and prevent new sample oxidation. Samples cannot be stored at -20°C. Once thawed from -70°C storage for assay, the sample should not be refrozen.

Samples should be protected from light to avoid photoxidation.

Sample preparation procedure

Tissue Homogenates

1. If necessary, remove blood by perfusion *in situ* with isotonic saline or *in vitro* by rinsing with ice-cold isotonic saline (i.e., 0.9% NaCl)
2. Weigh tissue
3. Prepare a tissue homogenate in 20 mM phosphate buffer, pH 7.4. Other buffers may be used, but the researcher should confirm non-interference in the assay by measuring the MDA and/or 4-HNE standards diluted in the chosen buffer.
4. Add 10 µL 0.5 M BHT in acetonitrile to 1 mL of tissue homogenate to prevent sample oxidation. A precipitate is expected. It will be removed by centrifugation and will not affect the outcome of the assay.
5. Centrifuge the homogenate to remove large particles (e.g., 3000 x *g* at 4°C for 10 minutes).
6. Remove an aliquot of the sample for protein determination.
7. Freeze the sample immediately at -70°C or keep on ice prior to testing. Test 0.2 mL of the homogenate in the LPO-586 assay.

Cell Culture

The protocol is similar for that of tissue homogenates. However, it is recommended that a homogenate of approximately 5 X 10^7 cells per mL be prepared for use in the assay. Cells cultured in serum containing medium should be washed several times to remove serum components prior to homogenization. Since MDA exists as the water soluble enolate anion at physiological pH, much of the MDA generated from lipid peroxidation in cell culture may be in the culture medium.

Plasma Or Serum

The amount of free MDA or HAE in normal plasma or serum is at or below the limit of detection of the LPO-586 assay.

Preparation of Standards

Malondialdehyde and 4-hydroxynonenal are provided as acetals because these compounds themselves are not stable. The acetals are hydrolyzed during the acid incubation step at 45°C, which will generate MDA (from TMOP) and 4-HNE.

The TMOP (S2) and 4-HNE diethylacetal (S1) standards are provided as 10 mM stock solutions. Dilute them 1/500 (v/v) in water just prior to use to yield a 20 µM stock solution for use in the assay. For a standard curve, pipette the volumes shown in table 1 to give a total of 200 µL of standard.

Table 1: Standard Curve Dilution Volumes

Target concentration of standard in reaction mixture, µM	**0**	**0.50**	**1.00**	**2.00**	**3.00**	**4.00**
Volume of 20 µM standard to add	0	25	50	100	150	200
Volume of water or buffer to add	200	175	150	100	50	0

ASSAY

Assay Procedure for MDA (Hydrochloric Acid Solvent Procedure)

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1. Prepare the standards according to Table 1 in clean glass test tubes or polypropylene microcentrifuge tubes. Preferably, standards should be run in triplicate.
2. Add 200 µL of sample to a clean glass test tube or polypropylene microcentrifuge tube. Unknowns should be run in triplicate.
3. Add 650 µL of diluted R1 reagent.
4. Mix gently by vortexing the sample.
5. Add 150 µL 37% (12 N) HCl.
6. Mix well and stopper the tube.
7. Incubate at 45°C for 60 minutes.
8. Centrifuge turbid samples (e.g., 15,000 x *g* for 10 minutes) to obtain a clear supernatant.
9. Transfer the clear supernatant to a cuvette.
10. Measure the absorbance at 586 nm.*

Assay Procedure for MDA + HAE (Methanesulfonic Acid Solvent Procedure)

In running an assay for the sum of MDA + HAE, OXIS recommends the use of MDA as the standard. Most studies that report concentrations of free MDA and HAE indicate an approximate 10-fold excess of MDA. Since the slopes of the calibration curves for MDA and HNE are not identical, some error is unavoidable in the determination of the two compounds together. However, the use of an MDA standard curve will minimize the error involved.

1. Prepare standards according to Table 1 in clean glass test tubes or polypropylene microcentrifuge tubes. Preferably, standards should be run in triplicate.
2. Add 200 µL of sample to a clean glass test tube or polypropylene microcentrifuge tube. Unknowns should be run in triplicate.
3. Add 650 µL of diluted R1 reagent.
4. Mix gently by vortexing the sample.
5. Add 150 µL R2 reagent.
6. Mix well and stopper the tube.
7. Incubate at 45°C for 60 minutes.
8. Centrifuge turbid samples (e.g., 15,000 x *g* for 10 minutes) to obtain a clear supernatant.
9. Transfer the clear supernatant to a cuvette.
10. Measure absorbance at 586 nm.*

*The color is stable for at least an hour at room temperature, or 2 hr at 4°C if the samples are stored in the dark and no evaporation occurs.

Sample Blank (Asb)

A sample blank should be measured to correct for any A_{586} contribution due to the sample. This blank is made by adding 650 µL of 75% acetonitrile/25% methanol instead of the diluted R1 reagent to the assay tube. The acid addition and sample incubation steps are then carried out as described above.

Reagent Blank (Ao)

A reagent blank, consisting of 200 µL of water, is included in the table describing the preparation of standards.

Standard Curve Characteristics

In both the HCl and methanesulfonic acid solvent procedures, the molar extinction coefficient (ε) at 586 nm for malondialdehyde is approximately 120,000. 4-HNE cannot be measured in the HCl protocol, as it does not form a chromphore with the reagent when HCl is the acid solvent. The color yield is a linear function of the MDA concentration over the range from 0 µM to 20 µM.

Calculations

1. Using the standard data, calculate the net A_{586} for each standard by subtracting the blank (A_0) value from each of the standard A_{586} values. Plot net A_{586} vs [MDA], and perform a linear regression analysis of A_{586} on [MDA]:

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$$[MDA] = a[MDA] + b$$

2. Calculate the concentration of analyte in each unknown from the net A_{586} of the sample (If a sample blank was required, subtract A_{sb} from the net sample absorbance.):

$$[\mathrm{MDA}] = \frac{A_{586} - b}{a} \bullet \mathrm{df}$$

Where:

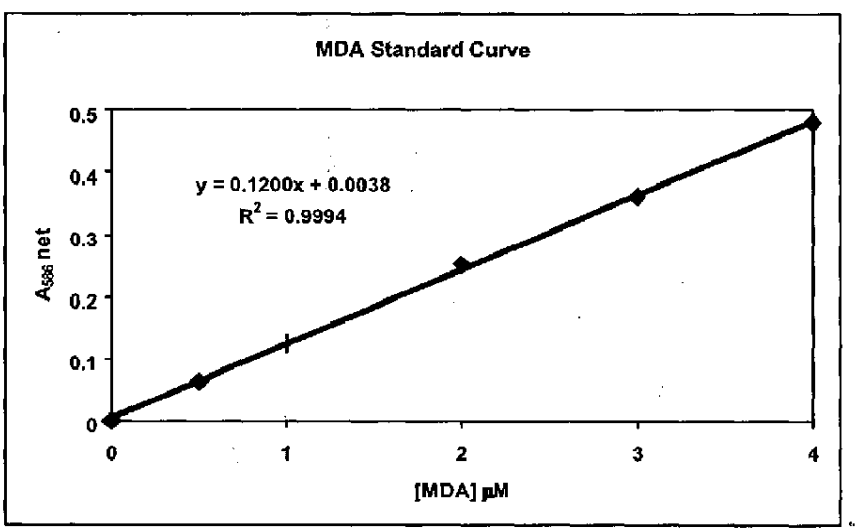
[MDA] is the µM concentration of MDA in the sample
A_{586} = Net absorbance at 586 nm of the sample
a = regression coefficient (slope)
b = intercept
df = dilution factor

Example

To illustrate the calculations, consider the following experiment:

Three 200 µL aliquots of a biological sample were assayed along with a triplicate set of MDA standards. A sample blank had zero absorbance. The average A_{586} value of the zero concentration standard was subtracted from the average A_{586} values of the other standards and the sample to give corrected absorbances (A_{586} net). A plot of A_{586} net vs. [MDA] for the standards was constructed (see below).

Figure 1: MDA Standard Curve



The sample had an average A_{586} net = 0.2752. Steps in the calculation of sample [MDA] are:

1. Calculate the [MDA] in the reaction mixture.

$$[\mathrm{MDA}] = \frac{0.2752 - 0.0038}{0.1200} = 2.261\,\mathrm{i\ M}$$

2. Correct for the dilution factor of the sample.

200 µL of sample was used and the final reaction volume was 1000 µL.

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$$df = \frac{1000}{200} = 5.000$$

[MDA] in biological sample = 2.261 µM x 5.000 = 11.3 µM

PERFORMANCE CHARACTERISTICS

Least Detectable Concentration

Experiments on enaldehyde standards and blanks suggest that the analytical limit of detection in a purified system will be 0.1 nmol/mL final concentration (0.5 nmol/mL in the sample), corresponding to an absorbance value of approximately 0.011. The limit for biological samples will generally be higher and it is recommended that the researcher assess this parameter in their system.

Reproducibility

Experiments in which standard samples (0-20 µM) were assayed using the same protocol over a period of 10 days established the standard error of the measurement (SEM) at less than 5%.

LPO-586 Assay in microtiter plates

For those who are interested in the application of the LPO-586 assay in microtiter plates, we provide the following information.

1. OXIS has not validated the use of the LPO-586 assay in microtiter plates.
2. The 45°C incubation and sample clarification (e.g., centrifugation) MUST be performed in stoppered test tubes/microcentrifuge tubes to ensure adequate mixing, a minimum loss of solvent due to evaporation, and successful sample clarification.
3. Measuring the absorbance of clarified samples in microtiter plates:
   a. The extinction coefficient for MDA/4-HNE will be different according to the effective path length in the particular microtiter plate reader.
   b. The absolute absorbance as a function of enaldehyde concentration will be lower, decreasing the assay sensitivity.
   c. If continuous wavelength monitoring is not available, 580 nm or 590 nm filters should be obtained to measure the samples.
   d. The user must establish that the particular microtiter plate to be used is chemically resistant to acetonitrile and strong acid.
4. Volume of the reaction mixture: A minimum of 0.5 mL total reaction mixture should be used to assure 0.2-0.3 mL of clarified sample be available for determining absorbance.
5. Once the conditions for measuring the absorbance of clarified samples in microtiter plates are established, they must be performed the same way each time.

NOTES

Limitations

1. Sucrose or fructose at concentrations of 50 mM or greater in the sample will cause a high bias in the assay. Vitamin E, when present at 15 µM or greater, can cause a diminution in the values obtained for the 4-hydroxyalkenals.
2. A pink color can sometimes be observed in samples or blanks when the methanesulfonic acid (R2 Reagent) protocol is used. This is due to the production of chromophores other than those producing the 586 nm peak. Ordinarily this pink chromophore will not interfere with the absorbance at 586 nm.
3. The LPO-586 assay measures only free malondialdehyde or 4-HNE in samples. The conditions of the assay do not provide for liberation of MDA bound to proteins via Schiff Base (5). 4-HNE is sufficiently reactive that it rapidly combines with proteins in tissues, forming

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stable adducts that are not liberated by heating at high temperatures in acid; as a consequence, there is very little free 4-HNE in tissue (1).

4. Normal tissues have very low levels of free malondialdehyde or 4-HNE, typically 10-100 pmol/mg protein (2,6). Assay of a 0.2 mL sample containing 10 mg of protein derived from normal tissue will give absorbance values at 586 nm of 0.01 or less in the LPO-586 assay. Caution must be taken not to interpret very low absorbance values (near zero) as an accurate reflection of analyte concentrations in biological samples.
5. In setting up the LPO-586 assay for the first time on a particular biological sample, the kinetics of color development on the sample should be followed in comparison with that of the TMOP or 4-HNE diethylacetal standards. The A_{686} of the sample should reach a plateau and then remain stable. If the A_{686} continues to go up after the standards have achieved a stable color, the researcher should be concerned that non-MDA reactivity (interference) is occurring in the sample.
6. In setting up the LPO-586 assay for the first time on a particular biological sample, a wavelength scan from 450 to 700 nm should be performed on the clarified sample reaction mixture and compared to that obtained with the TMOP or 4-HNE standard. The lack of a peak at 586 nm or lack of reasonable definition to the sample profile compared to the standard would suggest interference or non-specific reactivity in the sample.
7. If no antioxidant is added to the samples during homogenization and subsequent assay, a high bias due to new sample oxidation may result.
8. If turbid or hazy reaction mixtures do not clarify upon centrifugation, further efforts should be made to purify the sample. Turbid samples will give biased values in the assay.

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REFERENCES

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TECHNICAL SUPPORT

An OXIS Health Procucts, Inc. Technical Support Representative may be reached by telephone (800) 547-3686 Ext. 463, (503) 283-3911 Ext. 463, or e-Mail (techsupport@oxis.com) Monday through Friday 8:00 AM to 5:00 PM Pacific Time.

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